

A new time-scale for ray-finned fish evolution

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The Actinopterygii (ray-finned fishes) is the largest and most diverse vertebrate group, but little is agreed about the timing of its early evolution. Estimates using mitochondrial genomic data suggest that the major actinopterygian clades are much older than divergence dates implied by fossils. Here, the timing of the evolutionary origins of these clades is reinvestigated using morphological, and nuclear and mitochondrial genetic data. Results indicate that existing fossil-based estimates of the age of the crown-group Neopterygii, including the teleosts, *Lepisosteus* (gar) and *Amia* (bowfin), are at least 40 Myr too young. We present new palaeontological evidence that the neopterygian crown radiation is a Palaeozoic event, and demonstrate that conflicts between molecular and morphological data for the age of the Neopterygii result, in part, from missing fossil data. Although our molecular data also provide an older age estimate for the teleost crown, this range extension remains unsupported by the fossil evidence. Nuclear data from all relevant clades are used to demonstrate that the actinopterygian whole-genome duplication event is teleost-specific. While the date estimate of this event overlaps the probable range of the teleost stem group, a correlation between the genome duplication and the large-scale pattern of actinopterygian phylogeny remains elusive.

Keywords: Actinopterygii; dating; whole-genome duplication; fossils; fishes; phylogeny

1. INTRODUCTION

More than half of all vertebrates are ray-finned fishes (Actinopterygii; figure 1). The total number of species is now approaching 27 000, the vast majority of which are teleosts (Nelson 2006). Teleosts include numerous ecologically and economically important groups, as well as model organisms used for genomic and developmental biology research. Accurate estimates of actinopterygian divergence dates are therefore vital for addressing fundamental questions about the origin of modern vertebrate biodiversity and for making meaningful interpretations of experimental data. However, theories about the timing and pattern of the early evolution of ray-finned fishes are in a state of disarray and lag far behind our understanding of tetrapod evolution.

The Actinopterygii includes five major clades (figure 1): polypterids (e.g. bichir *Polypterus*); chondrosteans (e.g. sturgeon *Acipenser* and paddlefish *Polyodon*); lepisosteids (e.g. gar *Lepisosteus*); amiids (e.g. bowfin *Amia*); and teleosts (e.g. salmon *Salmo*). Polypterids are placed consistently as the sister group of all other ray-finned fishes, but deep conflicts exist between hypotheses of relationships among the other divisions. Morphology-based phylogenies (figure 1a) unite lepisosteids, amiids and teleosts as the Neopterygii (Regan 1923; Patterson 1973), although the identity of the neopterygian sister

group to the Teleostei is uncertain (Gardiner *et al.* 1996). Most nuclear gene analyses support a neopterygian topology (Le *et al.* 1993; Lecointre *et al.* 1993; Hoegg *et al.* 2004; Kikugawa *et al.* 2004; Crow *et al.* 2006), although, again, relationships within the Neopterygii are unstable. However, recent analyses of the nuclear gene *RAG-1* (Venkatesh *et al.* 2001) and complete mitochondrial genome sequences (Inoue *et al.* 2003) challenge the neopterygian hypothesis. Instead, both include a monophyletic group termed the ‘Ancient Fish Clade’ that unites chondrosteans, lepisosteids and amiids (figure 1b). Phylogenetic analysis of mitochondrial genomes yields strong support for the ‘Ancient Fish Clade’ topology in both Bayesian and maximum-parsimony statistical frameworks, although likelihood ratio tests of these same data are unable to reject neopterygian monophyly (Inoue *et al.* 2003). Other authors suggest that the ‘Ancient Fish Clade’ topology is an artefact of low taxon sampling (Cavin & Suteethorn 2006). This disagreement about deep actinopterygian phylogeny coincides with fresh interest in ray-finned fish evolution, much of which has been prompted by the discovery and subsequent analysis of an actinopterygian-specific whole-genome duplication event (Amores *et al.* 1998; Prince *et al.* 1998; Jaillon *et al.* 2004; Donoghue & Purnell 2005; Hurley *et al.* 2005; Crow & Wagner 2006; Mank & Avise 2006).

Predictably, morphology- and molecular-based estimates of the actinopterygian evolutionary time-scale also differ (figure 4). Molecular estimates are expected to exceed morphology-based dates, which are no more than

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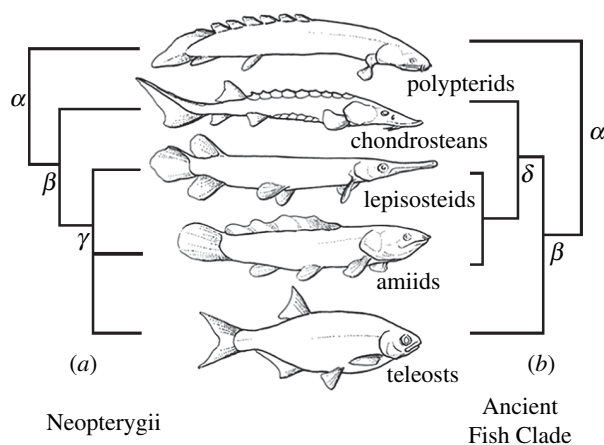


Figure 1. Alternative trees and group names of the major living actinopterygian clades: polypterids (bichir and reedfish); chondrosteans (sturgeons and paddlefish); lepisosteids (garpiki); amiids (bowfin); and teleosts. (a) Topology from morphological data, supporting the Neopterygii (Regan 1923; Patterson 1973). (b) 'Ancient Fish Clade' topology obtained from mitochondrial genomic data (Inoue *et al.* 2003; present analysis) and some nuclear genetic data (Venkatesh *et al.* 2001). α , the Actinopterygii; β , the Actinopteri; γ , the Neopterygii; δ , the 'Ancient Fish Clade'.

fossil-marked minima, but the incongruity is exceptional. Fossils place the minimum date for the origin of crown group teleosts at 151 million years (Myr) ago (Upper Jurassic; Arratia 2000), whereas recent mitochondrial genome data place the crown teleost date at around 285 and 334 Myr ago (Early Permian or Lower Carboniferous depending upon the dataset and the method applied; Inoue *et al.* 2005). Fossils date the earliest crown-group neopterygian at 245 Myr ago (Lower Triassic; Gardiner 1993; Grande & Bemis 1998), while mitochondrial genome estimates place the teleost divergence from amiids at around 417 and 390 Myr ago (Late Silurian or Middle Devonian depending upon the dataset and the method applied; Yamanoue *et al.* 2006). Successive fossil-based divergence estimates for the chondrosteian (crown Actinopteri) and polypterid (crown Actinopterygii) total groups date from 345 (Early Carboniferous) and 392 Myr ago (Mid-Devonian), respectively (Gardiner 1993; Dineley & Metcalfe 1999). However, comparable mitochondrial estimates do not yet exist, because node dates have been calculated using the 'Ancient Fish Clade' tree topology (Inoue *et al.* 2005). In summary, fossil data present extant actinopterygian diversity as the product of consecutive and widely separated pre-teleost radiations distributed throughout the latter half of the Palaeozoic and Mesozoic eras (figure 4). In contrast, molecular estimates embed all of these events well within the Palaeozoic, with even the teleost crown divergence occurring at least 35 Myr before the Mesozoic.

Given these extraordinary differences, the time is ripe for a new look at the evolution of ray-finned fishes. The aim of this paper is to examine three independent datasets composed of new and previously published data: (i) morphological material including fossils, (ii) four nuclear gene sequences, and (iii) complete mitochondrial genome sequences. These data are analysed separately, although calibrations for molecular-based node-date estimates are dependent upon fossil markers. We investigate the timing of primary events in basal actinopterygian phylogeny; we

search for congruent divergence date estimates derived from these different datasets; and we provide a new estimate of the phylogenetic location and date of the whole-genome duplication.

2. MATERIAL AND METHODS

(a) Taxa

(i) Morphological

Most morphology-based analyses of non-teleost actinopterygian phylogeny have examined Mesozoic and Palaeozoic taxa separately. Research has focused on the relationships within either crown Neopterygii (Gardiner *et al.* 1996; Arratia 1997; Grande & Bemis 1998; Cavin & Suteethorn 2006) or among Palaeozoic actinopterygians ('palaeoniscids') relative to chondrosteian and neopterygian stems and deeper parts of the actinopterygian tree (Gardiner & Schaeffer 1989; Coates 1999; Cloutier & Arratia 2004; Gardiner *et al.* 2005). Here, the dataset includes 29 fossil and Recent taxa, including representatives of all actinopterygian crown groups. In addition to many of the better-known Palaeozoic actinopterygians, a range of Mesozoic 'subholosteans', such as *Luganoia*, *Australosomus*, *Perleidus* and *Peltopterus*, are incorporated to increase the diversity of taxa bearing subsets of (generally accepted) neopterygian synapomorphies. Our taxon set bridges the Mesozoic-Palaeozoic divide and re-examines the phylogenetic position of two genera that we suggest are critical to the morphology-based underestimate of the age of the neopterygian crown node: *Brachydegma*, from the Early Permian (figure 2c-e; Dunkle 1939); and *Discoserra*, from the Lower Carboniferous (figure 2a,b; Lund 2000). *Brachydegma* has been grouped with *Acrolepis* (Schaeffer 1973), a stem actinopteran (Gardiner & Schaeffer 1989), and *Discoserra* has been considered either a stem polypterid (Lund 2000) or a stem actinopterygian (Cloutier & Arratia 2004). Character states for these taxa are described and discussed in figure 2 and the electronic supplementary material.

(ii) Molecular

A dataset consisting of sequences of four nuclear genes from 29 taxa was compiled. The taxon selection includes species covering chondrosteian, lepisosteid, amiid and teleost clades. Novel sequences generated for this study were combined with existing sequences taken from GenBank. Significantly, this study is the first analysis of the whole-genome duplication event to include critical representatives of all non-teleost clades, except polypterids. Previous studies lacked key groups (amiids, Hoegg *et al.* 2004; lepisosteids, Crow *et al.* 2006) and, in order to link the actinopterygian-specific whole-genome duplication event with the teleosts, have necessarily assumed monophyly of the Holostei (amiids plus lepisosteids). Database accession numbers of nuclear gene sequences are given in the electronic supplementary material, table 1.

The 33 mitochondrial sequences are from 28 genera, including all non-teleost actinopterygian clades. Novel sequences generated for this study were combined with existing sequences taken from GenBank. The overlap of non-teleost groups between the two datasets is of central importance, because the present analyses focus on the basal divergences within the actinopterygians. Database accession numbers of mitochondrial genomic sequences are given in the electronic supplementary material, table 2.

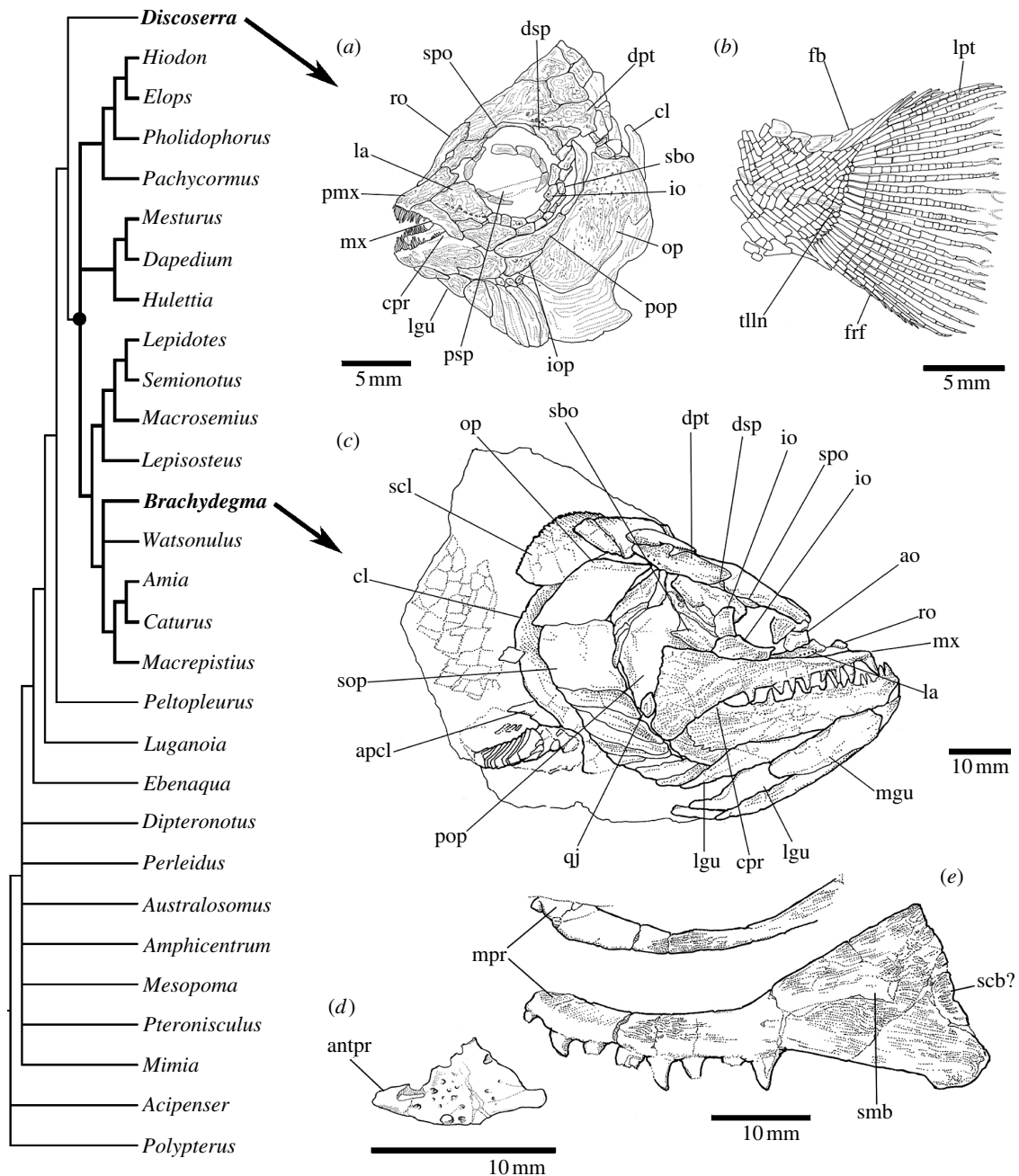


Figure 2. Strict consensus of the 116 shortest trees from the parsimony analysis of the morphological dataset of fossil and Recent Actinopterygii. Bold lines indicate crown-group Neopterygii. (a) *Discoserra pectinodon* Lund (2000), from the Mississippian (Serpukhovian) of Montana, USA. Dermal skull of CM (Carnegie Museum) 35211B in lateral view, anterior to left. Standard neopterygian apomorphies include the presence of an interopercular bone (iop), a supraorbital (spo), the shape and separation of the maxilla (mx) from the preopercular (pop), and the presence of a coronoid process (cpr) on the lower jaw. (b) *Discoserra pectinodon* (CM 41009A): caudal fin, external view with scales showing row-reversal at the meeting of peduncle and flank squamation. Neopterygian apomorphies include an abbreviated heterocercal shape (cf. modern lepisosteids), widely separated fin rays and the posterior terminus of lateral-line canal (tltn) directed onto fin, rather than extending posterior-dorsally along vestigial caudal lobe of the main body axis. Further details of braincase, hyoid arch, axial and caudal endoskeleton are provided in the electronic supplementary material. (c) *Brachydegma caelatum* Dunkle (1939), a basal stem-group halecomorph from the Early Permian (Artinskian) of Texas, USA. Skull and pectoral region of MCZ (Museum of Comparative Zoology, Harvard University) 6503 in lateral view, anterior to right. (d) *Brachydegma caelatum*, left antorbital, showing a fractured anterior process (antpr) and in-filled sensory canal. (e) *Brachydegma caelatum*, left maxilla in (upper) dorsal and (lower) lateral views. Apomorphies shared with *Amia* and its closest fossil relatives include large median gular (mgu), maxilla (mx) with indented rear margin and horizontal median shelf or process (mpr), and antorbital (ao) with tapering anterior process (antpr). Neopterygian apomorphies include the presence of supraorbital (spo), accessory postcleithrum (apcl) and coronoid process (cpr) on the lower jaw. antpr, anterior process; ao, antorbital; apcl, accessory postcleithrum; cl, cleithrum; cpr, coronoid process; dpt, dermosphenotic; dsp, dermosphenotic; fb, basal fulcrum; frf, fringing fulcrum; hp, hypurals; io, infraorbital; iop, interoperculum; la, lachrymal; lgu, lateral gular; lpt, lepidotrichia; mgu, median gular; mpr, mesial process; mx, maxilla; op, opercular; pmx, premaxilla; pop, preoperculum; qj, quadratojugal; qu, quadrate; ro, rostral; sbo, suborbital; scb?, possible scale-bone; scl, supracleithrum; smb, smooth unornamented band; sop, subopercular; spo, supraorbital; tltn, terminal lateral-line scale.

(b) Morphological data

The morphological matrix is based upon previously published data matrices (primarily Gardiner & Schaeffer 1989; Gardiner *et al.* 1996, 2005; Coates 1999); these were revised and augmented for the current taxon sample (see electronic supplementary material). The matrix includes 70 binary and multi-state characters; these were analysed under parsimony with the computer program PAUP* v. 4.0b10 (Swofford 1998).

Analysis of extant taxa alone (6 taxa) was completed using the exhaustive search option. Analysis of the complete taxon set (29 taxa) was undertaken using the heuristic search option with a parsimony 'ratchet' (Quicke *et al.* 2001). Two thousand replicates of random stepwise additions were carried out, followed by TBR (tree bisection–reconnection) branch swapping, holding only one tree in memory at any one time (i.e. MAXTREES = 1).

(c) Nuclear genetic data

Primers for *fzd8*, *hoxa11*, *sox11* and *tyr* were described previously (Chiu *et al.* 2002; Hoegg *et al.* 2004). Additional primers designed for the amplification of *hoxa11* were: HoxA11-5' CTTTTYTGCCCCAGACCCCGTCTTC and HoxA11-3' TGACGGTCRGTGAGGTTGAGCAG. Degenerate PCR conditions were described previously (Hoegg *et al.* 2004). Sequences were aligned based on the amino acid sequence; *fzd8*, *sox11* and *tyr* alignments follow those previously published (Hoegg *et al.* 2004). *Hoxa11* sequences were aligned using CLUSTAL X and adjusted manually. Regions for which reliable homology could not be established were excluded from all analyses.

Bayesian phylogenetic analyses were performed on the first and second codon positions of each gene; analyses were implemented using MRBAYES v. 3.04b (Huelsenbeck & Ronquist 2001). For each gene, the best-fitting nucleotide substitution model was selected using the Akaike information criterion (AIC) implemented in MrMODELTEST (v. 1.1b), modified from MODELTEST (Posada & Crandall 1998) by J. A. A. Nylander. Flat Dirichlet distributions were used for substitution rates and base frequencies, and default flat prior distributions were used for all other parameters. Metropolis-coupled Markov chain Monte Carlo analyses were run with one cold and three heated chains (temperature set to the default = 0.2) for 10 million generations and sampled every 1000 generations. Stationarity was confirmed by examining the plots of $-\ln L$ scores and parameter values; 8 million generations were discarded as burn-in. Single gene trees for *fzd8*, *sox11*, and *tyr* were rooted with the tetrapods *Homo sapiens*, *Mus musculus* and *Xenopus laevis*. *Hoxa11* was rooted with the shark *Heterodontus francisci*. Resulting individual gene phylogenies were examined to assign each sequence to its appropriate paralogue group (see electronic supplementary material, figures 5–8).

Species for which two or more genes could be unambiguously assigned to paralogue groups were included in a concatenated dataset of all four genes (2520 bp following exclusion of third codon positions). All concatenated sequences were formed by joining sequences from the same paralogue group and species, except for the combination of two different *Lepisosteus* species. A single paralogue from *Oncorhynchus mykiss* *hoxa11*, *Xenopus laevis* *fzd8* and *Xenopus laevis* *sox11* was chosen at random, as these paralogues are the result of more recent, lineage-specific duplication events. The concatenated dataset was divided into two data partitions (first and second codon positions). For each partition, the

GTR+I+ Γ model of nucleotide substitution was selected using the AIC implemented in MrMODELTEST (v. 1.1b). Bayesian phylogenetic analysis of the concatenated, partitioned dataset was performed as for the single gene analyses with 7.5 million generations discarded as burn-in. Maximum-likelihood analysis was performed on the concatenated dataset excluding third codon positions using PAUP* v. 4.0b10 (Swofford 1998). The GTR+I+ Γ model of nucleotide substitution was selected using the AIC implemented in MODELTEST (Posada & Crandall 1998). Bootstrap proportions for clades were assessed with 100 bootstrap pseudoreplicates. Maximum-likelihood analyses were also performed on concatenated amino acid sequences (1260 amino acids) in TREE-PUZZLE v. 5.2 (Schmidt *et al.* 2002) using the VT (Muller & Vingron 2000) substitution model and Γ -distributed rates. Amino acid frequencies and the Γ shape parameter α were estimated from the data. The Kishino & Hasegawa (1989) and Shimodaira & Hasegawa (1999) tests of an alternative phylogenetic topology including the 'Ancient Fish Clade' were performed on the concatenated nucleotide alignment using PAUP* v. 4.0b10 (Swofford 1998).

(d) Divergence date estimates using nuclear genetic data

Given the possible acceleration of evolutionary rates following genome duplication (Graur & Martin 2004), it was deemed inappropriate to estimate divergence dates from actinopterygian nuclear genetic data assuming a global molecular clock. Bayesian methods can allow for evolutionary rate variation among lineages and among genes in multi-gene datasets (Thorne & Kishino 2002). Here, these methods were used with multiple fossil calibrations (see electronic supplementary material). Minimum age limits were defined by the earliest fossil occurrence of a crown taxon. With the exception of the calibration for crown-group Actinopteri, no maximum age limits were imposed. The upper bound for this clade was estimated on the basis of the earliest unambiguous fossil remains of stem-group actinopterans (Middle Devonian), and it was necessary to make divergence date estimation analytically feasible. Previous estimates of actinopterygian divergence dates have used calibrations based on fossil and molecular data (Inoue *et al.* 2005; Yamanoue *et al.* 2006). As these molecular calibration data were dependent on the assumption of a global molecular clock, we have chosen to use only fossil calibrations.

The concatenated topology (figure 3) rooted with the tetrapods was used for the Bayesian divergence date estimation. The dataset was partitioned by gene. For each gene, PAML v. 3.14 (Yang 1997) was used to estimate the following model parameters under the F84+ Γ model of nucleotide substitution: nucleotide frequencies, transition/transversion parameter κ , and the shape parameter α of the discrete Γ distribution of rates among sites. The maximum likelihood of branch lengths and the variance–covariance matrix of branch length estimates were calculated using the program estbranches (Thorne & Kishino 2002). Divergence times were estimated using the program multidivtime (Thorne & Kishino 2002) and our set of fossil calibrations (see electronic supplementary material). The Markov chain Monte Carlo analyses were run for 10 million generations with a burn-in of 100 000 generations. The chain was sampled every 1000 generations. Convergence was verified by running multiple chains for each analysis. The following

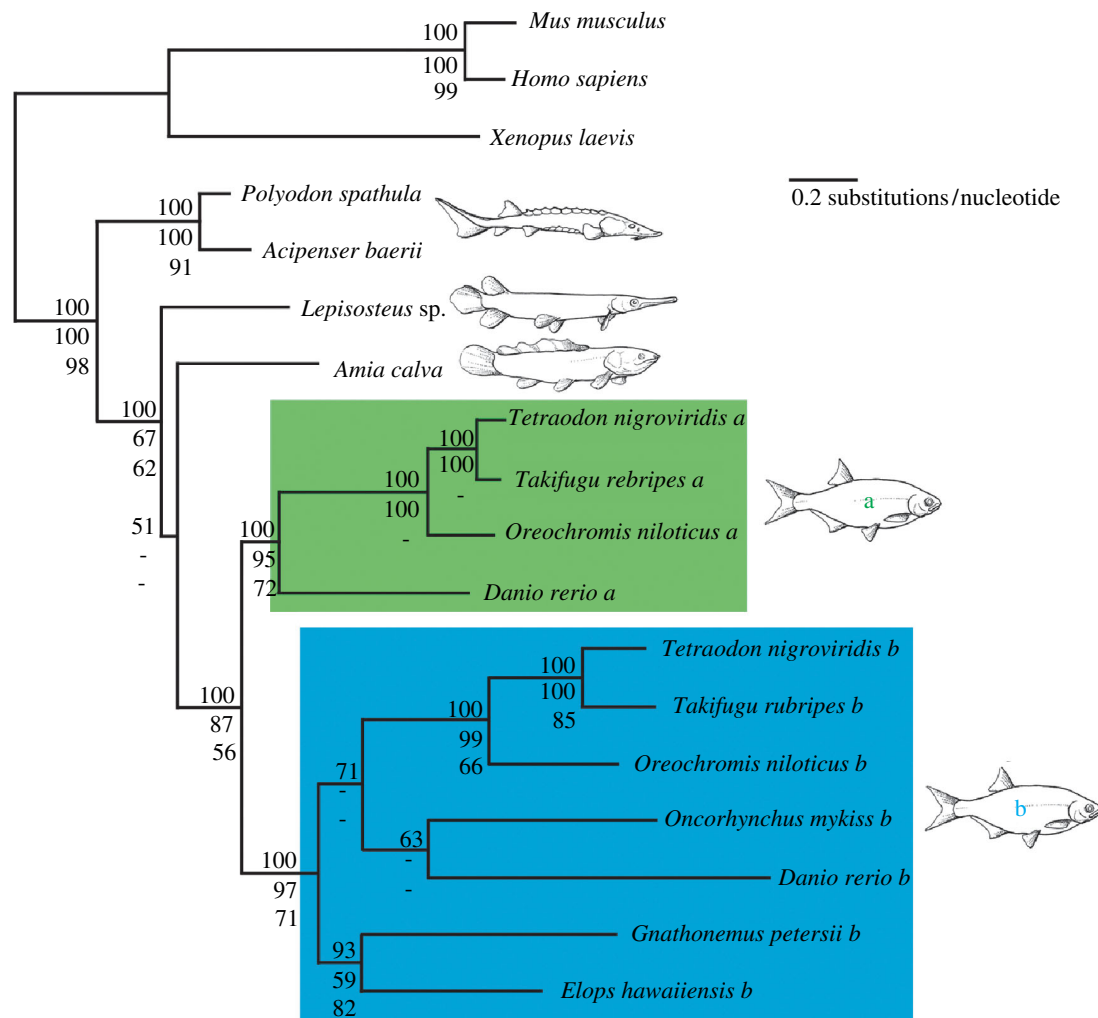


Figure 3. Phylogenetic relationships among actinopterygians inferred from partitioned Bayesian analysis of 2520 bp of concatenated nucleotide sequences of four nuclear genes (excluding third codon positions). All teleost concatenated sequences belong to either paralogue groups 'a' (green) or 'b' (blue), whereas amiid, lepisosteid and chondrosteian sequences fall outside of the teleost paralogue groups, indicating that the actinopterygian whole-genome duplication is specific to teleosts. The numbers on internal branches are (from top to bottom): Bayesian posterior probabilities and maximum-likelihood bootstrap proportions from nucleotide sequences, and quartet puzzling support values from amino acid sequences.

priors were used: mean time of the root, 3.7 (370 Myr ago; s.d. = 1.9); mean rate at the root, 0.030 substitutions per site per 100 Myr (s.d. = 0.015), which is the average mean distance from the in-group root to the tips divided by 370 Myr; and mean Brownian motion constant ν , 0.5 (s.d. = 0.5). Divergence dates were also estimated using these same parameters on a topology constrained to include a monophyletic Holostei.

(e) Divergence date estimates using mitochondrial genomic data

Divergence dates were re-estimated from mitochondrial genomic data using our fossil calibrations and a topology constrained to include the Neopterygii. This allowed direct comparison of mitochondrial and nuclear gene-based date estimates of key nodes. Not all nodes were directly comparable, because the taxa analysed were not identical between the two datasets. First and second codon positions of all protein-coding genes, except *ATPase8* and *ND6*, and stems of all tRNA genes, except *serine AGY*, were used for the divergence date estimation. The dataset was divided into three partitions: first codon positions; second codon

positions; and tRNA genes. Divergence dates were estimated as for the nuclear gene dataset with the following priors: mean time of the root, 4.1 (410 Myr ago; s.d. = 2.0); mean rate at the root, 0.050 substitutions per site per 100 Myr (s.d. = 0.025), which is the average mean distance from the in-group root to the tips divided by 410 Myr; and mean Brownian motion constant ν , 0.5 (s.d. = 0.5).

3. RESULTS

(a) Morphological data

(i) Phylogenetic analysis of extant taxa

The analysis of extant taxa alone yields one tree of 64 steps (41 characters informative for extant taxa; uninformative characters excluded; consistency index 0.81; retention index 0.75). The analysis provides robust support for neopterygian monophyly (figure 1a): 15 unambiguous apomorphies, with the collapse of this node requiring 12 extra steps. The Halecostomi (amiids plus teleosts) is also reconstructed as a clade, but support is weaker, with node collapse requiring only two extra steps. When trees were constrained to include the

‘Ancient Fish Clade’ (figure 1b), the tree length increased to 80 steps and clade support included only one unambiguous apomorphy.

(ii) *Phylogenetic analysis of extant and fossil taxa*

Analysis of the complete morphological dataset yields 116 trees of 234 steps (consistency index 0.47; retention index 0.7). A strict consensus of these trees (figure 2) shows a monophyletic Holostei (amiids plus lepisosteids), a monophyletic Neopterygii and a large polytomy at the base of the neopterygian stem group. When characters are reweighted (using rescaled consistency index and maximum value (best fit) options), a strict consensus of the three resultant trees conserves the branching structure of the consensus of unweighted results. In addition, the structure of the neopterygian stem group is mostly resolved, and a clade including *Hulletia*, *Dapedium* and *Mesturus* branches from the base of the teleost stem group. Of the taxa considered critical to this new analysis, *Discoserra* (formerly interpreted as either a stem polypterid or a stem actinopterygian) now branches from the apex of the neopterygian stem group. *Brachydegma* (formerly interpreted as a stem actinopteran) is placed as a stem-group amiid; therefore, it branches from within the neopterygian crown group. Many of the morphological synapomorphies supporting these results are illustrated in figure 2 and the electronic supplementary material, figure 1. In *Discoserra*, relevant conditions cover many parts of the skeletal anatomy (jaws, braincase, tail and axial skeleton); the resultant tree position is not the product of homoplastic change in a single morpho-functional character complex.

(iii) *Implications for divergences dates*

Brachydegma, dated at 284 Myr old, is now the fossil marker for the minimum date of the holostean crown group (the divergence of *Amia* from *Lepisosteus*), as well as the neopterygian crown group (figure 4). Under either the halecostome or the holostean hypotheses of neopterygian interrelationships, *Brachydegma* also establishes a minimum age for the teleost stem group.

Discoserra, dated at 318 Myr old, branches from the apex of the neopterygian stem group. Consecutive outgroups to *Discoserra* plus crown Neopterygii are each confined to the Upper Permian and Lower Triassic. The phylogenetic location of *Discoserra* therefore implies a series of taxon range extensions for the derived stem-group neopterygians passing from the Palaeozoic–Mesozoic boundary back into the Lower Carboniferous (figure 4).

(b) Nuclear genetic data

(i) *Phylogenetic analysis of nuclear genes*

The Bayesian and maximum-likelihood phylogenetic analyses performed on the concatenated nuclear genetic dataset provide significant support for a monophyletic Neopterygii (Bayesian PP=100%, maximum likelihood BP=67%; figure 3 and electronic supplementary material, figures 9,10). However, a monophyletic Halecostomi, in contrast to a monophyletic Holostei (Kikugawa *et al.* 2004), receives a low statistical support (Bayesian PP=51%, maximum likelihood BP<50%). When the nuclear gene topology is constrained to include a monophyletic ‘Ancient Fish Clade’, neither the Kishino & Hasegawa (1989) nor the Shimodaira & Hasegawa (1999)

test rejects this topology ($p=0.394$ and 0.199 , respectively). This indicates that nuclear genetic data provide an insufficient phylogenetic signal to distinguish unequivocally between the ‘Ancient Fish Clade’ and a monophyletic Neopterygii. Nevertheless, high statistical support for neopterygian monophyly in the phylogenetic analysis (Bayesian PP=100%, maximum likelihood BP=67%) suggests that teleosts, amiids and lepisosteids are all more closely related to each other than to the chondrosteans.

The nuclear gene analysis also corroborates the hypothesis that the actinopterygian whole-genome duplication occurred in the teleost stem group (Hoegg *et al.* 2004; Crow *et al.* 2006). All included teleost sequences belong to either paralogue groups ‘a’ or ‘b’ (figure 3), whereas amiid, lepisosteid and chondrosteans sequences fall outside the teleost paralogue groups and are therefore probably pre-duplication orthologues. *Hiodon alosoides* was excluded from the initial concatenated gene analysis, because paralogue identity could only be assigned to one of the four genes (*sox11*). However, *Hiodon* branches from close to the base of the teleost crown group; therefore, it is especially relevant for determining the phylogenetic placement of the whole-genome duplication event. For this reason, the concatenated analysis was repeated including the *H. alosoides sox11* data, with the three remaining *H. alosoides* genes coded as missing data. Results of this analysis also support a teleost-specific whole-genome duplication (see electronic supplementary material, figure 11).

(ii) *Bayesian analysis of node-date estimates from nuclear genetic data*

Divergence date estimates derived from nuclear genetic data are shown in figure 4 and listed in the electronic supplementary material, table 3. The date estimates for crown Teleostei and crown Neopterygii are 181–265 and 277–371 Myr ago, respectively. Inclusion of the newly re-diagnosed fossil *Brachydegma* in the calibrations yields very similar range extensions for Teleostei and Neopterygii (see electronic supplementary material, table 4). Divergence dates estimated on a topology constrained to include a monophyletic Holostei also yield similar results (see electronic supplementary material, table 5). The nuclear gene analysis yields a date estimate for the whole-genome duplication, placing the duplication event between 226 and 316 Myr ago (see electronic supplementary material, table 3).

(c) Mitochondrial genomic data: Bayesian analysis of node-date estimates

The phylogenetic analyses of mitochondrial genomic data yield a topology that contains a monophyletic ‘Ancient Fish Clade’ (see electronic supplementary material, figure 12). When the topological constraint of a monophyletic Neopterygii is enforced on the mitochondrial genomic data, lower-likelihood trees result from the maximum-likelihood phylogenetic analysis. However, this neopterygian arrangement is not rejected statistically using either the Kishino & Hasegawa (1989) or the Shimodaira & Hasegawa (1999) test (electronic supplementary material, table 6).

Divergence date estimates derived from mitochondrial genomic data, using a tree topology constrained to include

a monophyletic Neopterygii, are shown in figure 4 and listed in the electronic supplementary material, table 7. The 95% posterior credibility intervals for mitochondrial divergence dates are generally older than the divergence date estimates from nuclear gene data, although the two estimates overlap (figure 4). The date estimate for the crown teleost node is between 268 and 326 Myr ago, 50–100 Myr older than nuclear gene estimates. The date estimate for crown-group Neopterygii is between 327 and 378 Myr ago, up to 50 Myr older than nuclear gene estimates. The inclusion of *Brachydegma* as a fossil calibration does not alter the overall pattern of taxon range extensions (see electronic supplementary material, table 8). Divergence date estimates using a topology constrained to include a monophyletic Holostei yield similar results (see electronic supplementary material, table 9).

4. DISCUSSION

(a) *A new date for the neopterygian crown group*

The new minimum node date of 284 Myr ago (Lower Permian) for the neopterygian crown group (figure 4) marked by *Brachydegma* is some 39 Myr older than the previous standard fossil estimate. This places the neopterygian crown divergence deep within the Palaeozoic. An indirect corroboration is provided by the morphology of the 318-Myr-old *Discoserra*, which demonstrates that much of the morphological signature of crown Neopterygii had evolved by the end of the Lower Carboniferous (although the elevated phylogenetic location of *Discoserra* might be an artefact of its detailed preservation relative to more basally branching stem taxa). By drawing several additional advanced stem-group neopterygians known only from the Mesozoic into the Carboniferous (figure 4), *Discoserra* also indicates that the radiation of actinopterygians prior to the Upper Carboniferous has been underdetermined. It is noteworthy that, while the morphology-based analysis of extant actinopterygians delivers the halecostome pattern of neopterygian clades, the analysis including fossil taxa shows a monophyletic Holostei (see electronic supplementary material, figure 2). In fact, a monophyletic Holostei has been recovered elsewhere, in a recent analysis of nuclear genes (Kikugawa et al. 2004) and in an independent morphological study of fossil and extant fishes (Grande 2005).

This new minimum date for the crown Neopterygii is a radical departure from the current palaeontological consensus that neopterygian phylogeny concerns Mesozoic to Recent forms (Janvier 1996), but it aligns much more closely with molecular results. *Brachydegma* falls at the upper limit of the 95% posterior credibility interval (279–371 Myr ago; figure 4), resulting from the analysis of nuclear genes using independent fossil calibrations. Mitochondrial genomic divergence date estimates obtained using the same independent calibrations predict even older dates for the Neopterygii (327–378 Myr ago; figure 4), although they overlap with the estimates from nuclear gene data. In this respect, as well as identifying consistency between morphological and molecular data, our results highlight the value of applying a combined analysis of independent lines of evidence.

(b) *Causes of divergence date discrepancies*

Recognition of a fossil crown-group neopterygian (*Brachydegma*) in the Lower Permian and an advanced stem-group neopterygian (*Discoserra*) in the Mid-Carboniferous necessitates a series of taxon range extensions (ghost ranges) spanning the Permian and Upper Carboniferous (figure 4). An explanation is needed for the conspicuous underestimate of neopterygian divergence dates on the basis of palaeontological data. The erratic quality of the fossil record (including the likelihood of sampling rare taxa) is bound to influence reconstructed patterns of clade origination (Smith & Peterson 2002). In fact, Sepkoski's (2002) compendium of fossil marine animals reveals a major trough in actinopterygian diversity that extends throughout the Upper Carboniferous and the Lower and Middle Permian. Although it is possible that this pattern reflects a genuine biological signal, this seems unlikely. The limited number of marine ichthyofaunas spanning this interval indicates that this trough in actinopterygian diversity has arisen from a paucity of suitable fossil deposits. It follows that the currently accepted Mesozoic signal of a neopterygian radiation is probably an artefact of the exceptionally poor record of marine fishes during the preceding 70 Myr. In addition to this, the failure to find advanced neopterygians in this interval is compounded by the inability to recognize them. So-called 'subholostean' actinopterygians are among the least studied of all fossil vertebrates, and the informal persistence of this grade implies a series of uncharacterized stem groups. If limited fossil deposits throughout the Upper Carboniferous and the Lower and Middle Permian are largely responsible for the lack of pre-Mesozoic actinopterygian fossils, we would predict similar patterns of diversity through time in other marine clades. Future examination of additional taxonomic groups will support or refute this hypothesis.

In contrast to the neopterygian result, fossil evidence provides no support for the earlier crown teleost divergence dates suggested by analyses of nuclear (181–265 Myr ago) and mitochondrial (268–326 Myr ago) genes. An incomplete fossil record could be an explanation, but, in this case, the required date shift corresponds to no obvious trough in the number of taxa documented through time (Sepkoski 2002). However, it is noteworthy that, in North America at least, the number of sedimentary formations hits an all-time low at the Permo–Triassic boundary (251 Myr ago; Peters 2006). Alternatively, older dates might result from the artificial inflation of clade ages by analyses of one or both molecular datasets. Furthermore, like the neopterygian result, the mitochondrial divergence date estimate for the Teleostei is much older than the nuclear estimate. Other authors using mitochondrial sequences have obtained similarly anachronistic divergence times for the teleosts (Inoue et al. 2005; Peng et al. 2006; Yamanoue et al. 2006). In turn, these estimates are being used to constrain further molecular clock-based hypotheses of teleost clade divergences (Steinke et al. 2006). Corroboration for these dates can only be gained from vicariance biogeographic scenarios (and should be treated with caution; see Murray 2000), because the dates substantially predate any semblance of palaeontological support. Different selections of calibration markers are bound to underlie some of the resultant, controversial, date estimates, but in the present work the same set of fossil calibrations was used for both

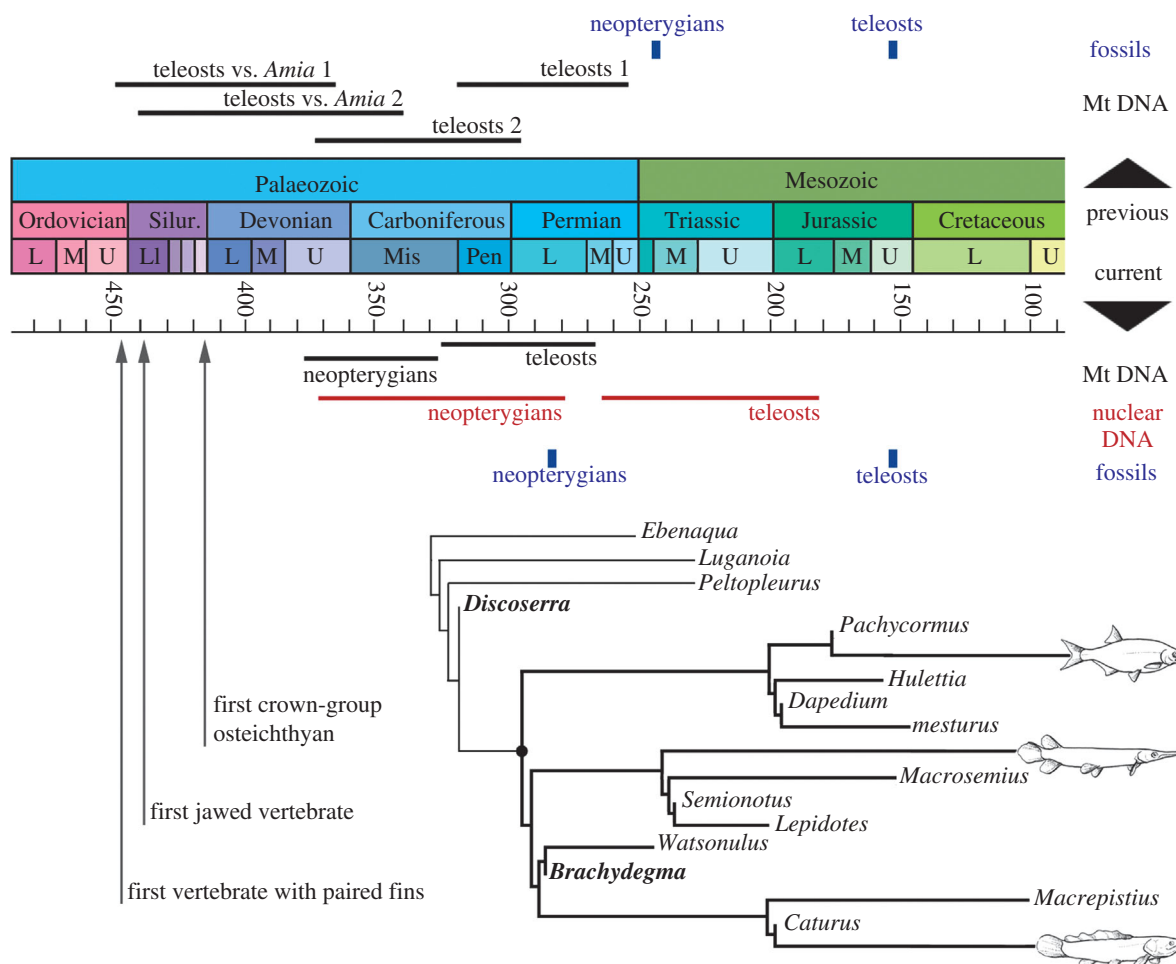


Figure 4. A new time-scale for actinopterygian evolution. A schematic providing comparison between teleost and neopterygian divergence date estimates derived from current and previous studies. Above geological column: previous fossil estimates (blue; Gardiner 1993; Grande & Bemis 1998; Arratia 2000) and previous mitochondrial genomic estimates (black; 1 or 2 depending on the method applied, Inoue *et al.* 2005; Yamanoue *et al.* 2006). Note a large disparity between divergence dates from previous fossil and mitochondrial datasets. Below geological column: divergence date estimates from the current study derived from fossil (blue), mitochondrial genomic (black) and nuclear genetic (red) data. Note congruent date estimates for the neopterygian divergence, but disagreement for the teleost date persists among different datasets. Phylogeny of Recent and fossil actinopterygians (simplified topology from the reweighted analysis of results in figure 2) plotted with divergence dates, illustrating the impact of *Discoserra* (Lund 2000) and *Brachydegma* (Dunkle 1939). Bold lines indicate the crown-group Neopterygii. Branch tips mark the earliest taxon occurrences in the geological record (subsequent taxon ranges not shown). Grey arrows juxtapose fossil dates for key events in vertebrate evolution (dates are conservative palaeontological estimates derived from Janvier (1996), Smith & Sansom (2001) and Long (2001); justification of the osteichthyan crown node can be found in the electronic supplementary material), highlighting discrepancies with previous molecular estimates of the teleost evolutionary time-scale. Geological column dates in Myr ago from Gradstein *et al.* (2004); series name abbreviations: L, Lower; Ll, Llandovery; M, Middle; Mis, Mississippian; Pen, Pennsylvanian; U, Upper.

nuclear and mitochondrial analyses. The discrepancy between our nuclear and mitochondrial node-date estimates suggests that such estimates of divergence times might be affected by evolutionary rate differences between these two genomes. However, differences in taxonomic sampling and tree topologies, particularly the inclusion of osteoglossomorphs in the mitochondrial dataset, might also be influential.

(c) Timing of the whole-genome duplication

Analyses that have used a global molecular clock place the actinopterygian whole-genome duplication (Jaillon *et al.* 2004) in excess of 300 Myr ago (350 Myr ago, Taylor *et al.* 2001; 350 Myr ago, Christoffels *et al.* 2004; 320 Myr ago, Vandepoele *et al.* 2004). Unless mitochondrial-based teleost divergence dates are accepted, these early Carboniferous dates conflict with a teleost-specific location in

phylogeny, and with the hypothesized link between the duplication event and the teleost radiation (Hurley *et al.* 2005; Donoghue & Purnell 2005; Mank & Avise 2006; Crow & Wagner 2006). Results of the present analysis confirm that the whole-genome duplication was a teleost-specific event (cf. Hoegg *et al.* 2004; Crow *et al.* 2006), but our data indicate that the duplication occurred significantly later than previously estimated (226–316 Myr ago). Combined with the revised node-date estimate of the neopterygian crown (fossil minimum 284 Myr ago; nuclear gene estimate 279–371 Myr ago), this analysis removes a major obstacle for the hypothesis that the whole-genome duplication was, in some sense, permissive to the teleost radiation. However, a 160+ Myr gap remains between the estimated duplication date and the prolific taxonomic and morphological diversification that occurred in a subset of teleost clades during the Late Cretaceous and early

Cenozoic. Furthermore, there is no obvious correlation between gene duplication and (actinopterygian) survivorship (Crow & Wagner 2006), because non-teleostean neopterygians flourish throughout the Mesozoic, following the end-Permian extinction event. Finally, we note that this emerging pattern of events in actinopterygian evolution contrasts strongly with the apparent coincidence of successive rounds of genome duplication and clade diversification within the Late Mesozoic and Cenozoic evolution of angiosperms (De Bodt *et al.* 2005).

(d) Concluding remarks

The findings of this research raise a number of issues that deserve further investigation. Actinopterygian diversity from the Devonian to the Triassic requires a thorough review, especially with regard to the prospect of discovering further cryptic examples of advanced stem group and primitive crown group neopterygians. Eurasian Permian marine shelf deposits might be especially productive in this regard. To a significant extent, the existing data gap and phylogenetic instability reflect lack of scientific attention. Molecular estimates of divergence dates that exceed fossil-based estimates by a significant amount cannot be dismissed out of hand (especially in the light of deficiencies in the documented fossil and sedimentary records), but close attention needs to be paid to the kinds of calibration applied. We also draw attention to the repeated discrepancies between results from mitochondrial and nuclear genetic data, and the possible effects of evolutionary rate differences. Finally, if the teleost-specific whole-genome duplication event did facilitate an increase in species and morphological diversity, the mechanism by which this was achieved remains elusive. Our data provide a new framework for future studies of the evolution of actinopterygian biodiversity.

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